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Effect of Lipid Composition and Liposome Size on Toxicity and In Vitro Fungicidal Activity of Liposome-Intercalated Amphotericin B

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Intercalation of amphotericin B into liposomes at a 10 mol% drug/lipid ratio decreased its cytotoxicity by 3- to 90-fold in cultured murine cells and reduced its lethality by 2- to 8-fold in a median lethal dose (LD_{50}) test in mice when compared with the commercial deoxycholate-solubilized drug (LD_{50} = 2.3 mg/kg). The cytotoxicity and lethality of the liposomal preparations were a function of their lipid composition and diameter. There was no correlation between the reduction of toxicity in the tissue culture assay and the reduction of lethality in the LD_{50} test. The rank order of reduction of lethality was sterol-containing liposomes > solid liposomes > fluid liposomes. In general, small sterol-containing vesicles were less lethal than large vesicles of the same composition. Intercalation of amphotericin B in sterol or solid liposomes increased not only the LD_{50} but also the time to death. The organ distribution of amphotericin B 24 h after intravenous administration was similar whether the drug was given as the commercial deoxycholate preparation or in liposomes. Finally, there were no differences among any of the formulations in their fungicidal activity against *Candida tropicalis* and *Saccharomyces cerevisiae* in vitro. The lesser and slower lethality of the liposome preparations coupled with the absence of any significant differences in organ distribution between the liposomal and detergent-solubilized drug suggests that the mechanism by which liposomes reduce the lethality of amphotericin B is by slowing its rate of transfer to a sensitive cellular target.

In the last decade a variety of novel methods have been proposed to improve drug delivery. Among these, the use of phospholipid vesicles or liposomes has received much attention, principally as a method to prolong drug levels following injection or to direct drugs to specific sites in the body (30). The advantage of liposomes as drug delivery systems stems from the biocompatible nature of the lipids used to form them and the ability to prepare them easily in various sizes and compositions (32).

Recently, it has been shown (27, 29) that amphotericin B, when intercalated into liposomes, is a more effective antileishmanial formulation than when given alone or as a deoxycholate suspension. The increase in efficacy was due to the diminished toxicity of the liposome amphotericin B preparations which permitted a greater dose of the drug to be given. Reduced toxicity of liposomal amphotericin B has been confirmed by a number of groups (4, 12, 16, 21, 33, 34). These investigators have also demonstrated that the liposomal formulations are more effective than deoxycholate dispersons of amphotericin B against fungal infections in animal models, presumably because lesser toxicity permitted larger doses to be given.

In the initial report (27), the lipid composition of the liposomal amphotericin B greatly influenced the toxicity of the formulation. We have confirmed that the lethality of the liposomal amphotericin B in mice is a function of the liposome composition. In addition, we have shown that liposome size or structure significantly modulates lethality. However, we found no correlation between the lethality of liposomal preparations of amphotericin B in vivo and the cytotoxicity of these preparations in tissue culture. To help elucidate the reasons for the lesser lethality of certain liposomal preparations, we have measured the tissue distribution in mice of amphotericin B injected either in liposomes or as the deoxycholate preparation. Finally, we have demonstrated that the antifungal activity in vitro of the liposomal amphotericin B is not affected by the compositions of the liposomes used in these studies.

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MATERIALS AND METHODS

Materials. Dimyristoylphosphatidylcholine, dimyristoylphosphatidylglycerol, dipalmitoylphosphatidylcholine, egg phosphatidylcholine (EPC), and oleoylpalmitoylphosphatidylcholine were obtained from Avanti Polar Lipids, Birmingham, Ala. Androsterone, ergosterol, and stigmosterol were purchased from Research Plus, Bayonne, N.J. Amphotericin B, cholesterol, cholesterylhemisuccinate (CHEMS), cholesterol sulfate, morpholine, and tocopherol acid succinate (TS) were obtained from Sigma Chemical Co., St. Louis, Mo. Fungizone, the commercial deoxycholate preparation of amphotericin B, was obtained from E. R. Squibb & Sons, Princeton, N.J., and was reconstituted with 5% dextrose immediately prior to use. The cholesterol was further purified by recrystallization from warm methanol twice. A chloroform solution of the TS was converted to the sodium salt by washing with 0.1 M sodium phosphate, pH 8.0. All other chemicals were of reagent grade and were used as received.

Liposome preparation. Liposomes were prepared as previously described (28, 34). For the dimyristoylphosphatidylglycerol (7:3) composition, the vesicles were prepared exactly as detailed by Lopez-Berestein and colleagues (21). Liposome size was determined by negative-stain electron microscopy as previously reported (28) or by laser light scattering on a light-scattering spectrophotometer (Malvern Instruments, Cherry Hill, N.J.). The Z average diameter and the polydispersity were determined as described before (34). A polydispersity index of 1.0 indicates a monodisperse population of particles, while values >1 indicate polydisperse populations of particles.

Drug analysis. The concentration of drug in the liposomes was determined as previously described (34). The phospholipid concentration was measured by the method of Bartlett (3).

Animal lethality testing: LD_{50} determination. Healthy Swiss Webster female mice (Simonsen, Gilroy, Calif.), 20 to 30 g, were injected via the tail vein with the amphotericin B liposome preparations diluted in sterile phosphate-buffered saline or with Fungizone diluted in 5% dextrose. The dose was adjusted for each animal on the basis of body weight. The mice were weighed every 24 h for 3 days. Deaths occurring within 1 h after dosing were considered immediate deaths. Mice that survived for 96 h invariably lived until sacrifice at 30 days. Median lethal doses (LD_{50}) were calculated by the method of Reed and Muench (31).

Toxicity in cell cultures. Amphotericin B as either the liposome (small unilamellar vesicles [SUV]) or Fungizone formulation was tested to determine the maximum dose tolerated without microscopic evidence of injury on a cultured murine macrophagelike cell line, RAW 264.7. Microtest plates were set up with 10^4 cells per 0.2 ml per well in H-21 Dulbecco modified Eagle medium containing 10% fetal calf serum and penicillin-streptomycin. The plates were incubated at 37°C in 5% CO_2. Twenty-four hours later, the cells were treated with either sterile SUV containing amphotericin B or Fungizone. The plates were read after 24 h. Each well was examined under 200-fold magnification for cell density and morphology. Visual comparison of the treated wells with nontreated control wells permitted the maximum tolerated dose to be assigned at the dilution at which no obvious decrease in cell density or changes in cell morphology were observed. Comparisons were also made of the effect of serial dilutions of drug-free liposomes of composition similar to that of the drug-containing ones.

Growth inhibition was studied with the LDP-1 murine fibroblast cell line. Culture dishes (35 mm) were seeded with 2×10^5 cells per 2 ml of H-16 Dulbecco modified Eagle medium containing 10% fetal calf serum and penicillin-streptomycin. The dishes were incubated at 37°C in 5% CO_2. The cells were treated after 24 h with (i) Fungizone, (ii) sterile SUV composed of EPC/cholesterol/TS (5:3:1) containing amphotericin B, (iii) drug-free SUV, or (iv) sodium deoxycholate in phosphate buffer to correspond to the amount of deoxycholate in the Fungizone preparation. Each of the four preparations was studied in triplicate. Twenty-four hours later, the cells were dislodged with 0.125% trypsin. Microscopic examination of the culture dishes revealed that all of the cells were removed by trypsin treatment. The number of cells in the dish was determined on a Coulter cell counter (Coulter Electronics, Inc., Hialeah, Fla.). The percentage of control cell growth was calculated with the following formula: % control growth = $[(N_{t,exp} - N_{0,exp})/(N_{t,con} - N_{0,con})] \times 100$, where N = the cell number; t = time of counting; 0 = time zero, when the drug was added; con = control, untreated cultures; and exp = treated cultures.

Antifungal activity. Two organisms were used to test the antifungal activity of amphotericin B: *Candida tropicalis* (ATCC 13803) and *Saccharomyces cerevisiae* (ATCC 9763). The MIC and minimum fungicidal concentration were determined as previously described (34).

Tissue distribution of amphotericin B and liposomes. Healthy female Swiss Webster mice were injected via the tail vein with Fungizone, amphotericin B SUV EPC/TS (7:3), or EPC/cholesterol/TS (5:3:1). The mice were sacrificed at various times after injection, and a 0.5-ml blood sample was removed via heart puncture. The liver, spleen, kidneys, and lungs were removed and weighed. Four mice were injected with each preparation for each time point. Amphotericin B was extracted from tissues by a methanol extraction procedure, and drug levels were determined by a high-pressure liquid chromatography method with *p*-nitrophenol as an internal standard (24). Standard curves for each tissue were constructed by "spiking" the relevant tissue and extracting the amphotericin B. Recoveries of amphotericin B from tissue samples spiked with the drug ranged from 50 to 71%.

Tissue distribution of the liposomes themselves, with and without amphotericin B, was assessed by labeling the liposomes with the iodinated lipid analog *p*-hydroxybenzamidinedihexadecylphosphatidylethanolamine (1). Mice were injected with the labeled liposomes at the same doses of lipids used in the amphotericin B distribution studies, and liposome distribution was determined as described previously (1).

RESULTS

Liposome preparations. The size distribution of the large multilamellar vesicles (LMLV) was quite heterogeneous, with a polydispersity index of >2.0 and an average diameter of >1.0 μm. The multilamellar vesicle (MLV) preparations had a polydispersity index ranging from 1.15 to 1.5 and average diameters ranging from 0.4 to 0.9 μm, depending on the lipid composition. The SUV had polydispersity indices of 1.07 to 1.18 and an average diameter between 0.06 and 0.14 μm, depending on the composition.

Sterile SUV (EPC/cholesterol/TS, 5:3:1) stored at 50 μmol/ml of lipid retained >98% of the amphotericin B as measured by chromatography on G-75 Sephadex after storage under nitrogen for 6 months. During this period the SUV gradually increased in size, from an initial diameter of 0.14 μm to a final diameter of 0.7 μm at the end of 6 months. This coalescence phenomenon was not observed in a control SUV preparation of the same composition but without amphotericin B. There was no significant alteration in size of the MLV and LMLV preparations stored under the same conditions. Nevertheless, for all of the studies, liposomes were prepared and used within 4 days.

Toxicity in tissue culture. A qualitative cytotoxicity assay consisting of the exposure of a macrophagelike cell line, RAW 264.7, to various concentrations of the amphotericin B preparations was performed. Cytotoxicity was apparent with the Fungizone at doses of 1.1 to 3.3 μg/ml. The liposomal amphotericin B resulted in less cytotoxicity than the Fungizone in all cases tested (Table 1). This reduction ranged from 3- to 90-fold. The vesicles which contained high molar ratios of TS appeared to be somewhat less effective at reducing the cytotoxic effects of amphotericin B than those without this lipid.The preparation that consistently gave the best reduction in toxicity in vitro was composed of EPC alone, which, as shown below, was not the case in vivo.

It was often observed that the cytotoxic effect of drug-free liposomes was greater than that of the amphotericin B liposomes of the same composition when compared at the same lipid concentration (data not shown).

As another measure of cytotoxicity, the growth-inhibitory effect of amphotericin B dispersed in either deoxycholate (Fungizone) or liposomes (EPC/cholesterol/TS, 5:3:1) was quantitated in a cell growth assay. This composition was chosen because of its ease of preparation and good reduction of lethality in vivo. The concentration required to cause a 50% growth inhibition (median inhibitory concentration

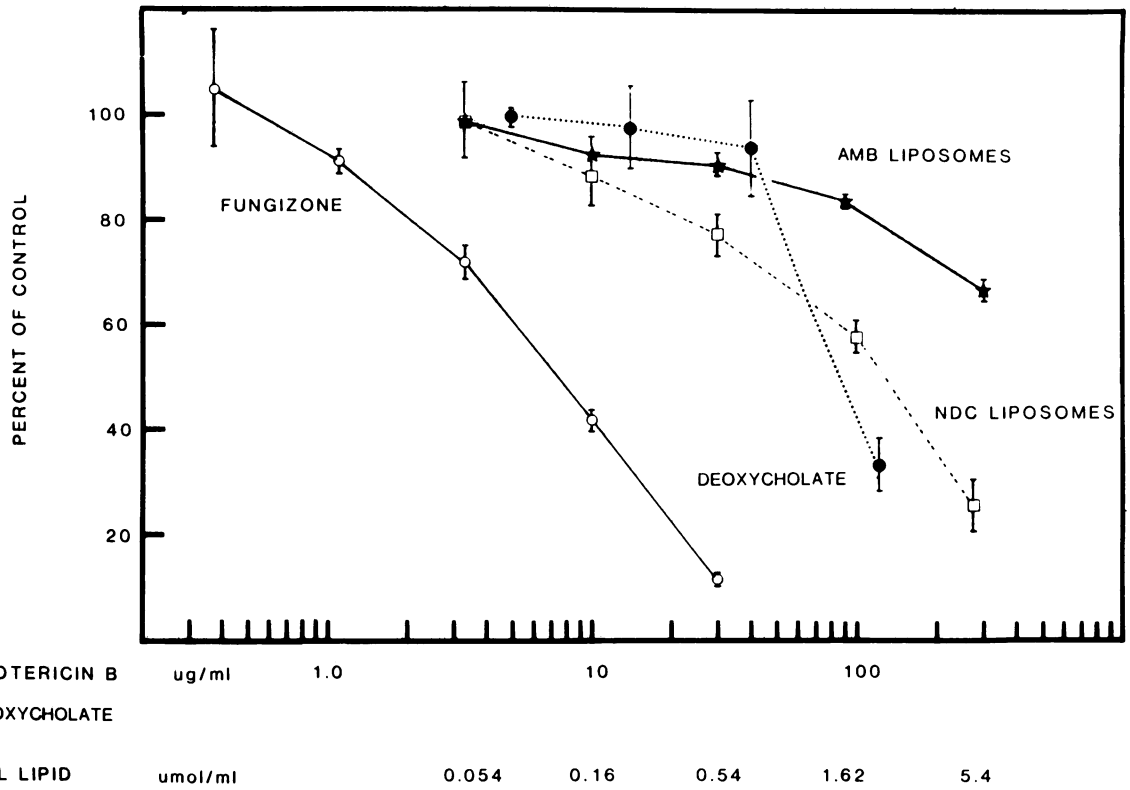

FIG. 1. Effect of Fungizone, amphotericin B (AMB) liposome, deoxycholate, or non-drug-containing (NDC) liposomes on growth of cultured murine L cells. Symbols: (○) amphotericin B concentrations when added as Fungizone; (●) deoxycholate concentrations; (□) non-drug-containing liposomes, lipid concentrations; (★) amphotericin B concentrations when added as SUV (EPC/cholesterol/TS, 5:3:1). Each value is the mean ± standard deviation of three separate determinations.

[IC_{50}]) was about 60 times greater for the liposome formulation (IC_{50} > 300 μg/ml) than for Fungizone (IC_{50} = 7.5 μg/ml) (Fig. 1). The IC_{50} for the deoxycholate alone was about 90 μg/ml (0.23 μmol/ml), whereas that for the lipid alone was 2.0 μmol/ml. Thus, drug-free liposomes had a growth-inhibitory effect which was reduced by intercalation of amphotericin B.

In this growth inhibition assay neutral drug-free liposomes, EPC/cholesterol (5:3) (IC_{50} = 10 μmol/ml), were fivefold less inhibitory than negatively charged drug-free liposomes composed of EPC/cholesterol/TS (5:3:1) or EPC/cholesterol/egg phosphatidylglycerol (9:8:1) (IC_{50} = 2.0 μmol/ml).

TABLE 1. Ratio of maximum tolerated dose of liposomal amphotericin B to Fungizone amphotericin B on murine RAW 264.7 cells

Drug prepn	Composition	Molar ratio	Fold reduction in cytotoxicity	
			Expt 1	Expt 2
Fungizone			1	1
Fluid liposomes	EPC	1	90	30
	EPC/TS	7:3	90	10
Sterol liposomes	EPC/TS/androsterone	5:4:1	10	3
	EPC/TS/cholesterol	5:4:1	10	10
	EPC/TS/ergosterol	5:4:1	10	3
	EPC/TS/stigmosterol	5:4:1	10	10
	EPC/cholesterol/TS	5:4:0.1	30	30
	EPC/cholesterol/CHEMS	5:3:1	30	30

In vitro lethality. The tissue culture assay is a convenient tool to estimate the protective effect of dispersing amphotericin B in liposomes. However, to take into account the biological factors which might affect toxicity, we measured the lethality of various liposomal amphotericin B formulations in mice. When amphotericin B in EPC/cholesterol/TS (5:3:1) SUV was administered to female Swiss Webster mice, the LD_{50} (13.3 mg/kg of body weight) was considerably greater than the LD_{50} obtained for the Fungizone preparation (2.3 mg/kg of body weight; Fig. 2 and Table 2). Similar LD_{50}s were found for these same preparations in male Swiss-Webster mice and for Fungizone and EPC/TS (7:3) SUV in CD-1 female mice (Charles River Breeding Laboratories, Inc., Lexington, Mass.).

Each of the liposome preparations we studied exhibited some protective effect against the lethality of amphotericin B in mice. Sterol-containing liposomes were the most effective, "solid" liposomes containing saturated phospholipids such as dipalmitoylphosphatidylcholine, with high transition temperatures, were intermediate, and "fluid" liposomes composed of lipids with transition temperatures well below 37°C, the body temperature of the mouse, were the least effective in protecting against the acute toxicity of amphotericin B.

Not only was the acute toxicity reduced, but the time of death was affected by liposome intercalation (Fig. 2). For example, in the animals receiving Fungizone, a twofold increase in amphotericin B concentration from 1.5 to 3.0 mg/kg resulted in death of 60% of the animals immediately

TABLE 2. Effect of composition on LD_{50} of liposomal amphotericin B in mice

Composition[a]	Prepn	Molar ratio	No. of animals	LD_{50} (mg/kg)			
				1 h	24 h	48 h	72 h
Amphotericin B-deoxycholate (Fungizone)	1		100	2.6	2.6	2.4	2.3
Fluid liposomes							
EPC	2	1	18	5.0	5.0	4.3	4.3
EPC/TS	3	7:3	100	9.0	8.0	4.4	3.8
OPPC/TS	4	7:3	18	4.5	4.5	4.5	4.5
DMPC/DMPG	5	7:3	40	>12	9.2	4.8	4.2
Solid liposomes							
DPPC/TS	6	7:3	24	>15	>15	11.7	8.9
Sterol liposomes							
EPC/TS/androsterone	7	5:4:1	18	4.7	4.7	4.5	4.4
EPC/TS/cholesterol	8	5:4:1	18	12.8	12.8	12,3	11.8
EPC/TS/ergosterol	9	5:4:1	18	>20	15.2	15.2	14.1
EPC/TS/stigmosterol	10	5:4:1	24	>20	20	17.8	17.8
EPC/cholesterol/TS	11	5:3:1	132	>30	25	13.8	13.3
EPC/cholesterol	12	5:4	48	>35	30	20.9	19.0
CHEMS	13	1	31	>30	20.6	17.7	17.7
Cholesterol sulfate	14	1	18	>15	>15	>15	>15

[a] OPPC, Oleoylpalmitoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; DPPC, dipalmitoylphosphatidylcholine.

after injection; an additional 22% of the animals died by day 4. By contrast, a twofold increase of the liposomal amphotericin B from 9 to 18 mg/kg resulted in no immediate deaths but rather a gradual accumulation of deaths to day 4. After day 4 there were no additional deaths with any of the preparations examined in this study. The pattern of a more gradual onset of the lethality of amphotericin B was observed with all of the sterol-containing and solid liposomal formulations, but not with the fluid liposomes (Table 2).

To define the relationship of the sterol structure to the reduction of lethality, we examined the influence of four sterols on the median lethal dose of liposomal amphotericin B (Table 2, preparations 7 to 10). The sterols were incorporated at a 10 mol% ratio in a liposome composed of EPC/TS (5:4), a composition that easily forms SUV upon sonication; this ensured that differences in size were not a confounding factor in the interpretation of the data. The rank order in the reduction of lethality of the preparations was stigmosterol > ergosterol > cholesterol >> androsterone (Table 2). When the mole ratio of cholesterol was increased beyond that in preparation 8 (Table 2), the liposomal amphotericin B became even less toxic (preparation 11); the least toxic preparation was SUV composed of EPC/Chol (5:4) (preparation 12; LD_{50} = 19.0 mg/kg). Liposomes prepared from CHEMS, an analog of cholesterol with a succinate moiety attached to the three position, also were highly effective at reducing the toxicity of amphotericin B (LD_{50} = 17.7 mg/kg), as were liposomes composed of cholesterol sulfate (LD_{50} = 15 mg/kg). The disadvantage of the CHEMS composition is that drug-free CHEMS liposomes have an LD_{50} of 1 to 1.5 g/kg, whereas most of the other compositions tested had LD_{50}s for the drug-free liposomes of >4 g/kg.

In addition to the composition, the size or structure of liposomes also plays a significant role in their effect on the acute toxicity of amphotericin B in mice. For sterol-containing liposomes, the greater the diameter, the greater the lethality of the formulation (Table 3). This was found to be the case even for the CHEMS preparation, a single-component composition that has a high ratio of sterol/

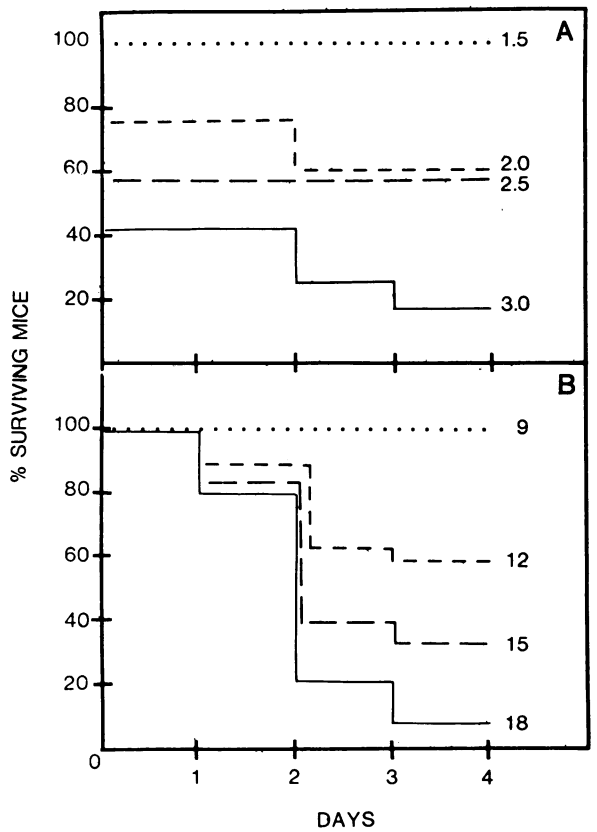

FIG. 2. Survival of mice given amphotericin B intravenously. Amphotericin B given as the (A) Fungizone preparation or (B) the liposomal preparation, SUV (EPC/cholesterol/TS, 5:3:1). The value to the right of the line in both panels indicates the dose in milligrams of drug per kilogram of body weight. Each dose was administered to 24 animals.

TABLE 3. Effect of vesicle type on LD_{50} of liposomal amphotericin B in mice

Composition	Molar ratio	Type of liposome	No. of animals	LD_{50} (mg/kg)			
				1 h	24 h	48 h	72 h
Fluid liposomes							
EPC/TS	7:3	SUV	18	4.6	4.6	4.3	4.3
		MLV	24	>8.0	>8.0	7.2	6.9
		LMLV	24	>8.0	>8.0	7.2	6.9
DMPC/DMPG[a]	7:3	SUV	40	>12	9.2	4.8	4.2
	Nonheated	LMLV	48	5.9	5.6	4.4	3.8
	Heated	LMLV	24	>18	>18	9.8	9.8
Sterol liposomes							
EPC/cholesterol	5:4	SUV	48	>35.0	30.0	20.9	19.0
		MLV	23	18.6	16.0	14.1	12.2
		LMLV	36	17.5	16.9	11.8	8.6
EPC/TS/stigmosterol	5:4:1	SUV	24	>20.0	20.0	17.8	17.8
		MLV	18	>20.0	18.4	14.9	10.0
		LMLV	18	>18.0	>18.0	16.4	15.0
EPC/cholesterol/TS	5:3:1	SUV	48	>18.0	>18.0	14.0	13.4
		MLV	54	25.7	14.3	8.8	8.2
CHEMS	1	SUV	31	>30.0	20.6	17.7	17.7
		MLV	31	18.0	14.2	13.3	12.4

[a] DMPC, Dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol.

amphotericin B, making it unlikely that a phase separation is the factor accounting for the increase of lethality with size. In the most extreme example, EPC/cholesterol (5:4), changing the preparation from SUV (0.09 μm) to the LMLV (2.5 μm) increased the lethality by a factor of 2.2. Since the larger liposomes were also multilamellar, we were not able to distinguish whether it was size alone or structure (number of lamellae) which was the more important factor. By contrast, in the liposome preparations lacking cholesterol (EPC/TS, 7:3), an increase in liposome size was associated with a slight decrease in lethality.

We also examined the lethality of a dimyristoylphosphatidylcholine/dimyristoylphosphatidylglycerol (7:3) LMLV (16) and observed an LD_{50} of 3.8 mg/kg when the preparation was maintained at a temperature of no higher than 20°C until the time of injection. This formulation has been reported to have an $LD_{50} > 12$ mg/kg, albeit in a different strain of mouse (16, 21). When the preparation was warmed at 37°C for 30 min prior to being injected into mice, an LD_{50} of 9.8 mg/kg was obtained. Thus, it appears that composition and size or structure are not the only parameters influencing the lethality; rather, for certain formulations the conditions used to prepare and store the liposomes are important.

In vitro fungicidal activity. There was no appreciable difference in either the MIC or the minimum fungicidal concentration between the liposome amphotericin B and the Fungizone (Table 4). Drug-free liposomes at a concentration comparable to that used in the drug-containing liposomes had no cytotoxic effects on the two fungal strains as measured in the MIC or the minimum fungicidal concentration.

In vivo disposition of amphotericin B. One possibility to account for the reduction in lethality observed upon incorporation of amphotericin B into liposomes is that the distribution of amphotericin B in the body is altered following injection in this drug carrier so that susceptible tissues are protected. The tissue distribution of amphotericin B after intravenous injection was determined by an internally standardized high-pressure liquid chromatography assay (24). Two SUV compositions containing amphotericin B were studied. The first composition consisted of EPC/TS (7:3),

TABLE 4. In vitro antifungal activity of amphotericin B and liposomal amphotericin B

Prepn	*C. tropicalis* (ATCC 13803)			*S. cerevisiae* (ATCC 9763)		
	No. of tests	μg/ml (SD)		No. of tests	μg/ml (SD)	
		Mean MIC	Mean fungicidal concn		Mean MIC	Mean fungicidal concn
Amphotericin B (Fungizone)	8	0.94 (0.33)	1.80 (0.78)	6	0.37 (0.21)	0.47 (0.17)
Fluid liposomes						
EPC/TS (119:1)	5	0.88 (0.34)	1.25 (0.77)	5	0.44 (0.17)	0.44 (0.17)
EPC/TS (7:3)	5	0.88 (0.34)	1.00 (0.34)	5	0.31 (0)	0.44 (0.17)
Sterol liposomes						
EPC/TS/androsterone (5:4:1)	4	0.78 (0.31)	1.25 (0)	5	0.31 (0)	0.31 (0)
EPC/TS/cholesterol (5:4:1)	5	1.13 (0.28)	1.75 (0.69)	5	0.50 (0.17)	0.50 (0.17)
EPC/TS/stigmosterol (5:4:1)	5	0.75 (0.28)	1.50 (0.56)	5	0.31 (0)	0.38 (0.14)
EPC/TS/ergosterol (5:4:1)	5	1.50 (0.56)	2.00 (0.69)	5	0.31 (0)	0.44 (0.17)
EPC/cholesterol/TS (5:4:0.1)	5	1.25 (0)	1.75 (0.69)	5	0.44 (0.17)	0.50 (0.17)
EPC/CHEMS/TS (5:4:0.1)	5	0.81 (0.42)	1.0 (0.34)	5	0.31 (0)	0.56 (0.14)

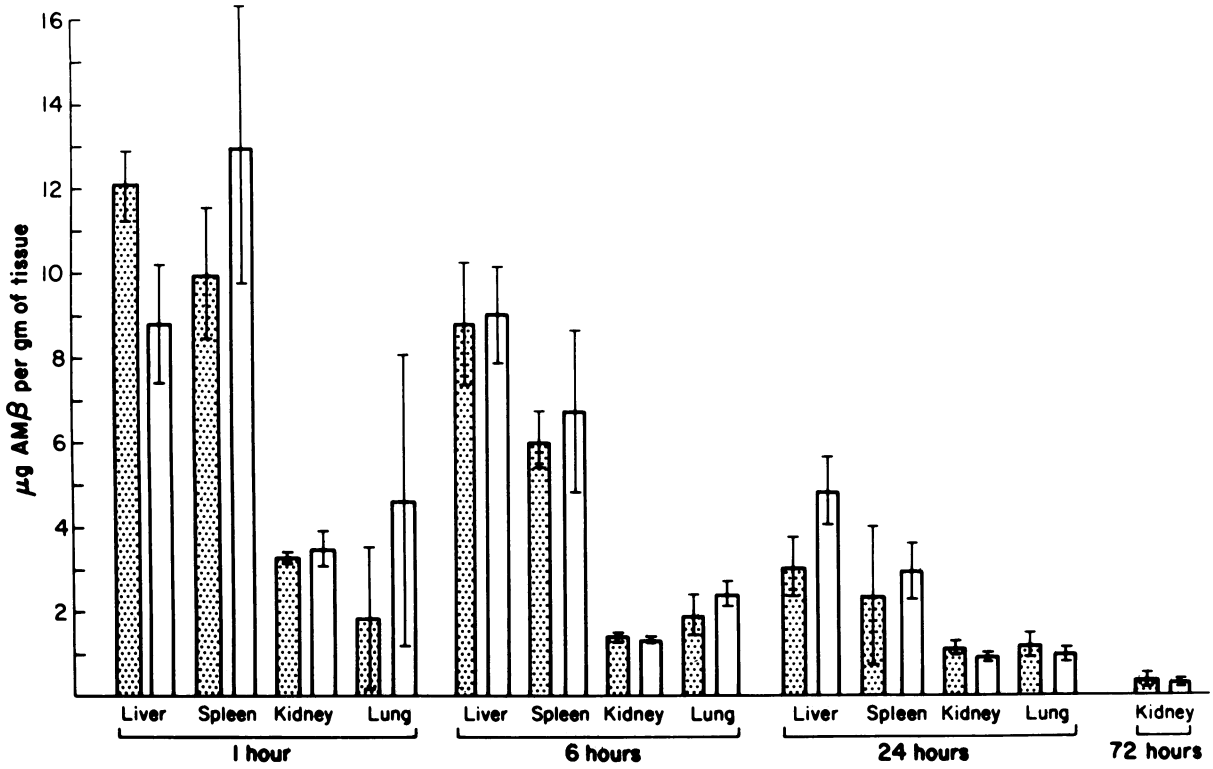

FIG. 3. Concentration of amphotericin B (AMB) in selected tissues of Swiss Webster mice following intravenous injection of Fungizone or liposomal amphotericin B. Symbols: open bars, amphotericin B administered as Fungizone at a dose of 1 mg/kg; shaded bars, amphotericin B administered as SUV (EPC/TS, 7:3) at a dose of 1 mg/kg. For both preparations each value is the mean ± standard deviation of tissue concentration in four animals.

which reduced the lethality of the drug by 1.7-fold (Table 2). The second composition consisted of EPC/Chol/TS (5:3:1), which reduced the lethality of the drug by 5.8-fold (Table 2).

Injection of SUV (EPC/TS, 7:3) containing amphotericin B resulted in a tissue distribution of drug that was almost identical to that observed for the Fungizone (Fig. 3). The greatest amount of drug from either preparation was found in the liver. On a per gram of tissue basis, however, the spleen received as high a dose as the liver. At 72 h the residual drug concentration in the kidney was similar for both preparations.

Since the SUV (EPC/TS, 7:3) reduced the toxicity of amphotericin B by only 1.7-fold, we compared the hepatic concentration of amphotericin B given as Fungizone or in a more protective formulation (EPC/Chol/TS, 5:3:1) (Table 5). The initial levels of amphotericin B in the liver were again similar with Fungizone and liposomal amphotericin B. However, the concentration of amphotericin B injected as Fungizone showed a continuous decrease in the liver with time, whereas the concentration of amphotericin B injected as SUV (EPC/Chol/TS, 5:3:1) remained fairly constant between 6 and 96 h after injection (Table 5).

Disposition of liposomes in vivo. The distribution of the liposomes themselves following intravenous injection was

TABLE 5. Concentration of amphotericin B in liver after intravenous, administration

Time postinjection (h)	Concn (μg/g of liver)[a]	
	Amphotericin SUV	Fungizone
1	7.2 ± 1.7	8.2 ± 1.5
3	5.2 ± 1.8	8.2 ± 0.6
6	3.1 ± 0.6	4.6 ± 0.4
12	3.8 ± 0.5	3.8 ± 0.2
24	4.4 ± 0.8	2.9 ± 0.4
48	5.0 ± 0.9	2.7 ± 0.3
96	2.6 ± 0.2	<1
192	1.9 ± 0.3	<1

[a] SUV composition was EPC/cholesterol/TS (5:3:1). Total concentration of amphotericin B injected was 1 mg/kg of body weight. Concentrations are the mean ± standard deviation (n = 4).

TABLE 6. Disposition of ^{125}I-labeled SUV at 1 h after intravenous injection in mice

Organ	% of injected dose (mean ± SD, n = 4)	
	SUV[a]	Amphotericin B SUV
Liver	42.9 ± 5.7	46.4 ± 5.9
Spleen	2.5 ± 0.6	1.4 ± 0.3
Lung	0.7 ± 0.1	0.6 ± 0.1
Kidney	1.6 ± 0.1	1.6 ± 0.4
Heart	0.5 ± 0.1	0.6 ± 0.2
Carcass[b]	28.0 ± 3.2	24.1 ± 2.0
Blood	16.9 ± 2.7	14.7 ± 2.9

[a] SUV composition was EPC/cholesterol/TS (5:3:1). The total lipid injected was 25 μmol/kg of body weight. The total radioactivity injected was 3.1 × 10^6 dpm of ^{125}I-labelled lipid per kg of body weight.

[b] Carcass, Remainder of animal after removal of listed organs.

examined by labeling the lipid bilayer with the nonexchangeable ^{125}I-lipid label *p*-hydroxybenzamidinedihexadecylphosphatidylethanolamine (1). The distribution of SUV composed of EPC/Chol/TS (5:3:1) was compared with that of the liposomes containing 6.8 mol% amphotericin B in the bilayer. One hour after injection of 25 μmol of lipid per kg of body weight, which in the drug-containing formulation carried 1.7 mg of amphotericin B per kg of body weight, there was no significant difference in distribution of the lipid between the two formulations (Table 6). When the lipid dose was increased fivefold, the distribution of liposomes was similar to that observed at the lower dose and, again, there was no difference in distribution between liposomes with or without amphotericin B (data not shown).

DISCUSSION

The experiments reported here were designed to define the mechanism responsible for the reduction of lethality of amphotericin B intercalated in liposomes. In all systems tested, amphotericin B intercalated into liposomes was less toxic than that solubilized in deoxycholate. Lipid composition and size of structure of the liposomes were important determinants of the extent of reduction in toxicity. However, the effect of liposome composition on toxicity was not consistent between the in vivo lethality model and the in vitro cytotoxicity model in that fluid nonsterolic liposomes appeared to be more protective than sterol-containing liposomes in tissue culture whereas the reverse was true in the animals. Our results suggest a mechanism by which liposomes moderate amphotericin B toxicity and reinforce the importance of animal tests in new drug development; these two topics will be discussed in turn.

Mechanism of the reduction of amphotericin B toxicity by liposomes. There are three principal mechanisms by which liposome intercalation might reduce the acute lethality of amphotericin B: (i) it might change the gross organ distribution of amphotericin B in the animal, thereby diverting the drug from its lethal targets; (ii) it might alter the fundamental nature of the interaction between amphotericin B and its lethal receptors; or (iii) it might alter the rate at which amphotericin B reaches its lethal receptors. Clearly, all three explanations relate to the interaction between the drug and certain receptors which are presumed to mediate acute toxicity. Although more than one mechanism may apply, our results suggest that the third one of these explanations is likely to be the most important in the reduction of lethality of amphotericin B following intercalation in liposomes.

Distribution of amphotericin B and liposomes. At the dosages studied, there did not appear to be a major difference in the initial tissue distribution of amphotericin B in four major organs when administered in Fungizone or SUV formulations (Fig. 3; Table 5). Conversely, the disposition of the liposomes, as measured by the lipid label, was not appreciably affected by the incorporation of amphotericin B into the lioposomes (Table 6). The disposition in mice of amphotericin B given in either form appears to be similar to that observed in humans when Fungizone is administered (10). These observations make it unlikely that the decrease in lethality of amphotericin B in the liposomal form is due to a gross alteration in tissue distribution of the drug.

Fundamental drug-receptor interaction. The toxicity of amphotericin B is due to a permeability defect in membranes as result of the formation of an amphotericin B-sterol complex (17). Selectivity for fungal membranes is due to a preferential interaction of the drug with ergosterol, the principal sterol of fungal membranes, over cholesterol, the principal sterol of mammalian membranes (25). We found no appreciable difference in antifungal activity between Fungizone and any of the liposome formulations of amphotericin B. Previously, we have shown that, microgram for microgram, amphotericin in SUV was as efficacious as Fungizone in a *Candida* infectious mouse model (34). Thus, liposome intercalation has not altered the fundamental interaction of amphotericin B with ergosterol, its receptor in the fungal membrane. It seems unlikely that the intrinsic interaction of amphotericin B with a putative cellular receptor in the mammalian cells would be altered by incorporation of the drug into liposomes.

Our results differ in one respect from those reported by Hopfer and colleagues (13). They found that amphotericin B in MLV containing ergosterol were considerably less fungicidal than in MLV lacking ergosterol. The difference may be due to the presence of TS in our formulations which would act as a fluidizing agent (19) and promote the transfer of the amphotericin B from the liposome into the fungal membrane. Other differences are the lower mole ratio of ergosterol in our liposomes and our use of SUV rather than MLV. Only 5 to 10% of the lipid is on the external monolayer of MLV as opposed to 50 to 60% in SUV (32). Amphotericin B does not rapidly cross bilayers (35); thus, it might be that the drug cannot transfer as readily from internal lamellae in MLV as from SUV.

Rate of transfer of amphotericin B to receptors. The data in Table 2 reveal that the reduction of lethality of the liposome formulations in vivo, when size is held constant, is a function of lipid composition: in rank order of protective effect, sterol liposomes > solid liposomes > fluid liposomes. Of the sterols, ergosterol and stigmosterol were more effective at reducing the toxicity than cholesterol was. These measurements of the LD_{50} confirmed the previous reports of decreased lethality of amphotericin B in liposomes (27).

We also found that the kinetics of lethality were substantially different when the drug was incorporated into liposomes. This suggests that the rate of transfer of the amphotericin B to critical receptor is decreased when the drug is incorporated into liposomes. The slower rate of transfer would lead to less damage and might permit the damage to be repaired. At low concentrations of amphotericin B, membrane damage, as assessed by potassium efflux from hamster cells, is reversible (22, 23).

A considerable amount is known concerning the thermodynamics of binding and the kinetics of transfer of amphotericin B in bilayer systems. This information is useful in understanding the observed pattern of lethality of liposomal amphotericin B. From equilibrium binding techniques the apparent affinity coefficients for the binding of amphotericin B to lipid membranes are as follows: ergosterol-containing membranes > cholesterol-containing membranes > solid membranes (those lacking sterol and composed of synthetic phospholipids at a temperature below the transition temperature of the phospholipid) > fluid membranes (membranes lacking sterol and composed of phospholipids at a temperature above the transition temperature) (6, 37). Clearly, the rank order of these affinity coefficients correspond exactly to the rank order of mitigation of toxicity of amphotericin B by liposomes made of these various lipids.

Lipid composition also significantly affects both the kinetics of transfer of amphotericin B into the bilayer and the type of amphotericin B-lipid complex formed (2, 5, 6, 8, 14, 36, 37). Bolard and his colleagues (6) examined the rate of exchange of amphotericin B between liposomes and demon-

strated that exchange occurs through the aqueous phase and not by collisional transfer or fusion. They found that amphotericin B was transferred rapidly from vesicles in the fluid state but not from those in the solid state (6).

Thus, lipid compositions of the kind known to bind amphotericin B more avidly or transfer the drug more slowly appear in our studies to reduce the toxicity of amphotericin B to a greater extent than do other lipid compositions. This observation, in conjunction with the disposition data, leads us to suggest that liposomes alter the rate at which amphotericin B reaches a cellular receptor associated with toxicity.

In vitro cytotoxicity. In the tissue culture experiments the liposomal amphotericin B was 3- to 90-fold less toxic to the mammalian cells than the Fungizone. Our results for both the deoxycholate and liposome amphotericin B agree with those of previous studies. Others have shown that, after 24 h of incubation, the growth of baby hamster kidney cells was significantly reduced by 5 μg and totally inhibited by 15 μg of amphotericin B per ml as the deoxycholate dispersion (22). For L-929 and HeLa cells, viability was decreased after a 2-h incubation with 40 μg of Fungizone per ml as assessed by trypan blue exclusion (18). About 6 μg of Fungizone per ml is sufficient to induce erythrocyte hemolysis in vitro, but more than 100 μg/ml is required when the amphotericin B is incorporated into liposomes (26).

An interesting finding is that amphotericin B-containing liposomes were less inhibitory to cell growth than drug-free liposomes. The effect of the drug-free liposomes was anticipated; it has been shown previously that lipid vesicles added to cell cultures at concentrations >0.5 μm/ml can inhibit cell growth (7). It is difficult to explain the protective effect of amphotericin B. Low concentrations of amphotericin B can increase uridine incorporation (18) and stimulate cell proliferation (25). It might be that the decrease in growth rate caused by large amounts of lipid vesicles in the culture can be compensated for by a growth-stimulating effect of low concentrations of amphotericin B.

The liposomal drug formulations were less toxic than Fungizone in both the in vitro and in vivo toxicity tests; however, the effect of lipid composition in the two systems was not consistent. In vivo, sterol-containing liposomes were the least toxic (Table 2), whereas in vitro non-sterol-containing liposomes were the least toxic (Table 1). The superiority of the non-sterol-containing composition in vitro could be due to the ability of such liposomes to remove cholesterol from the cell membrane (9), thus making the cell less sensitive to the effects of amphotericin B. Whether a similar result would be observed in other in vitro toxicity tests such as K^+ efflux measurements (22, 23) or erythrocyte hemolysis (26) is not known. However, it is clear that the tissue culture cytotoxicity test cannot replace the in vivo assay for predicting the lethal dose response to liposomal amphotericin B.

Liposomes as a carrier for amphotericin B. Liposomal amphotericin B is a promising improvement over the current formulation of the drug. Indeed, encouraging results have been published on a liposomal amphotericin B formulation in humans (20). It is obvious from our results that a number of details concerning liposome preparation and storage influence the extent of the reduction of lethality. For instance, when the lipid composition was held constant, the size of the vesicles had an appreciable effect on the LD_{50}. For the sterol-containing preparations the lethality increased as the vesicle size increased. In the case of the dimyristoylphosphatidylglycerol/dimyristoylphosphatidylcholine (7:3) LMLV preparation, the LD_{50} varied depending upon the history of the preparation. These results illustrate that formulation of amphotericin B in liposomes for use in humans must take into account a number of characteristics not usually encountered in antimicrobial therapeutic agents; a commercial formulation of liposomal amphotericin B will have to be physically as well as chemically stable.

Finally, it should be emphasized that we have tested acute but not long-term toxic effects of the liposome formulations. Because tissue disposition is function of size, and because LMLV preparations are distributed to the lung to a greater extent than SUV (15), it would not be surprising to find size-related differences in chronic toxicity between the different formulations. We would anticipate that SUV generally would be less toxic than LMLV preparations.

In summary, amphotericin B intercalated in liposomes is less toxic than, but as efficacious as, the drug solubilized in deoxycholate. The decrease in toxicity when SUV are the carrier is suggested to be due to a reduction in the rate of transfer of the drug from the liposome into cellular membranes and not to a major redistribution of the drug in the organs of the body tissues. Liposomal amphotericin B is a promising improvement over the current formulation of the drug and merits further development as an antifungal agent.

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